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(54) Title: RADIO-LABELLED ANTIBODIES FOR IMAGING

(57) Abstract

Scintigraphic detection thrombi in mammals can be effected by injecting a solution of a radio-labelled agent which can be a material from a reconstituted lyophilised kit and then labelled e.g. with technetium-99m. The agent is produced from a starting material from the group consisting of proteinaceous materials, monoclonal antibodies, single domain antibodies or an epitope binding fragment of monoclonal antibodies or single domain antibodies. The starting material is selected as one specifically directed against the blood clots and thiolating is effected for example with DL N-Acetylhomocystein-thiolactone. An important embodiment is one in which the thiolation step produces the Fab' fragment.

* See back of page

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RADIO-LABELLED ANTIBODIES FOR IMAGING

The present invention relates to radio-labelled antibodies or other proteinaceous materials for imaging.

More specifically, the present invention is concerned with scintigraphic detection of thrombi in mammals including humans. However, substances and processes developed to facilitate detection of thrombi may also have usefulness for other imaging such as of tumours.

It has already been proposed to label various proteins for antibodies with radiometal ions for scintigraphic or therapeutic applications. Labelling has been with technetium-99m because of its advantageous physical properties. Of particular interest has been the use of monoclonal antibodies raised against specific antigens. When such antibodies are labelled with radiometal ions, they can localise specifically to their antigens and therefore such antibody conjugates can be used as diagnostic or therapeutic tools.

Specific labelling of antibodies with technetium-99m has been described using a disulphide bond reducing agent such as dithiothreitol (DTT) to expose sulphydryl groups on the antibodies for binding to technetium-99m (see for example European patent specification no. 0-237150 and PCT specification W088/07382).

However, it is considered there is a need to develop new and useful processes and products which are economic, simply and easily practiced and which will provide an appropriate vehicle capable of being labelled with a suitable radionuclide such that the vehicle will relatively rapidly and effectively preferentially locate at the site of a blood clot whereby effective imaging can take place.

The present invention concerns developments in this field which offer at least a n w and useful alternative to known proposals.

According to one aspect of the present invention, there is provided a method of producing a kit for scintigraphic detection of thrombi in mammals including humans, the steps comprising taking a material which is directed specifically against blood clots, the material being from the group consisting of a proteinaceous material, a monoclonal antibody, a single domain antibody, or an epitope binding fragment of a monoclonal antibody or a single domain antibody, and conjugating the material with a thiolating agent, whereby there is provided a conjugated material adapted to be labelled with an acceptable radionuclide.

It has been found that by conjugation with a thiolating agent, it is possible at least with some embodiments of the invention to provide a high level of labelling efficiency whereby a most effective kit for detection of thrombi can be produced. Labelling efficiencies as high as 100% are possible.

Surprisingly it has been found that at least preferred embodiments of the invention produce a labelled antibody or fragment which effectively and rapidly preferentially locates in thrombi. This greatly facilitates effective scintigraphic imaging.

Preferably, the monoclonal antibody is Mab 3 B6/22 (3B6). Monoclonal antibody 3B6 recognises the D-dimer (DD) epitope of human cross-linked fibrin. Monoclonal antibody 3B6 is available from AGEN Biomedical Limited, of Brisbane, Queensland, Australia amd is described in Australian patent no. 572,125.

A preferred radionuclide is ^{99m}Tc (technetium-99m) because of various advantageous physical properties such as being a pure gamma emitter of 140 keV, short half life, and being readily available. How ver, other radionuclides might be useabl such as rhenium. Rhenium has several isotopic

forms namely 186, 188, 189 and 191 and is a beta and gamma emitt r and similarity of chemical characteristics with technetium-99m makes it a candidate for use in conjugating to protein carriers. Rhenium-protein conjugates may be useful for therapeutic applications.

It has been found that the present invention can be advantageously implemented where use is made of the Fab' fragment of a suitable monoclonal antibody. The Fab' fragment can be produced by (i) pepsin digestion of the antibody to produce the $F(ab')_2$ fragment and then (ii) this fragment is reduced by a suitable reducing agent such as dithiothreitol (DTT) to produce the Fab' fragment. It is thought that this step activates internal thiolated groups and makes the groups receptive to subsequent processing. Reduction, however, may be implemented without using DTT. By using a reducing agent such as DTT to reduce disulphide bonds in a fragment such as $F(ab')_2$, the smaller fragment Fab' can be obtained but it is now pointed out that this is a reversible process. By contrast the present invention makes use of a thiolating agent (with or without the preliminary step of reduction with an agent such as DTT) and most significantly it has been found that the use of the thiolating agent is especially beneficial in suppressing or preventing Fab' fragments recombining to form $F(ab')_2$. Furthermore enhanced labelling efficiency was found to occur by using the thiolated Fab' fragments of the antibody. Furthermore it has been found that an initial reduction step with an agent such as DTT is unnecessary.

After the use of dithiothreitol as reducing agent, treatment with a thiolating agent and the final purification of the labelled antibody, analysis of a radio-labelled antibody was made to determine the extent of technetium incorporation as well as the effect on the immunoreactivity of the antibody as a function of reducing agent concentration. A high level of technetium incorporation occurred at a reducing agent/antibody mole ratio of 12:1.

Increased radiolabel incorporation probably reflects on the reduction of disulphide bridges around the antibody hinge region. The process had little effect on the immunoreactivity of the antibody under the relatively low concentrations of reducing agent used.

Although a preferred embodiment of the invention consists in using the Fab' fragment of the antibody due to enhanced labelling, the invention also extends to labelling the antibody and other fragments thereof including $F(ab')_2$. When dealing with problems of the vascular system such as thrombolic disorders, it is believed the Fab'_2 fragment will give the best results for scintigraphy. The Fab' fragment has a relatively small size which allows clot penetration together with rapid blood clearance and this will provide an excellent target-to-blood ratio well suited for scintigraphic detection.

Preferably, and especially where the Fab' fragment is used, the thiolating agent DL N-Acetylhomocystein-thiolactone is used.

Preferred embodiments of the invention may include exchange labelling of the thiolated antibody or reduced fragment, for example by using radionuclide labelled gluconate and preferably a purification step follows, for example by the use of gel column chromatography or high pressure liquid chromatography (HPLC). The resultant product is then ready for injection.

An advantageous embodiment of the invention produces a three vial kit ready for use with radionuclide labelling which takes place just before use. The vials are produced as follows:

Thiolated Fragment Vial

A $F(ab')_2$ fragment of a monoclonal antibody specific to thrombi is prepared by a known method. The fragment is thiolated to produced the Fab' fragment and purification takes place to provide a source of thiolated fragment which

can then be freez d dried and stablised into the vial. Thiolation is best carried out with the use of a suitable catalyst. Thiolation of proteins and specifically antibodies is known, as is the use of catalyst (see for example Warzynski et al, J. Immunol Methods 35, 157-168, 1980). Routine experimentation is used to determine the precise process conditions to suit the particular antibodies. It is thought that a low degree of thiolation of antibodies can be achieved via ε-amino groups without affecting the N-terminal α-amino group. This is believed to avoid affecting the binding site of the antibody, thereby avoiding deterioration of the immunoreactivity and avidity of the antibody.

Surprisingly, in an embodiment of the invention in which $F(ab')_2$ was thiolated, instead of obtaining thiolated $F(ab')_2$, the smaller fragment Fab' was obtained. It is believed that the thiolation breaks down the $F(ab')_2$ fragment leaving the Fab' fragment carrying both exogeneously bound sulphydryl groups and endogenous sulphydryl groups due to breaking down of disulphide bonds of $F(ab')_2$. The mechanism of this reaction is not known. It has been found that the resultant thiolated Fab' has significant advantageous qualities compared to Fab' generated by reductive cleavage using DTT. The advantages include the following:

(a) Thiolated Fab' fragments contained exogeneously added sulphydryl groups which are believed to bind most effectively technetium-99m.

(b) The Fab' fragment, chemically modified by the thiolating step, appears to possess more than one and perhaps up to three endogenous sulphydryl groups in close array on the antibody and these are believed to be particularly suited for stable binding of technetium-99m.

(c) It is believed that the binding of technetium-99m is by virtue of both the endogenous sulphydryl groups pr sent on the polypeptide backbone of the antibody fragment Fab' and the exogeneously added sulphydryl groups.

(d) No evidence of recombination to $F(ab')_2$ is seen from the thiolated Fab' when lyophillised into kits and this is a most significant difference compared to DTT generated Fab'. This may be due to thiolated Fab' having modified protein confirmations which do not favour recombination. However, the chemistry of the process is not well understood. It is believed that recombination to $F(ab')_2$ will greatly reduce the ability of the antibody to bind technetium-99m as a result of reduced availability of free sulphydryl groups.

(e) It has been found that labelled thiolated Fab' kits have produced only a slight affect on the immunoreactivity of the fragment.

Buffer Vial

A vial of a suitable buffer is provided for adding to the freeze dried thiolated fragment prior to labelling.

Imaging Agent Vial

A suitable renal or hepatic imaging agent is provided such as Sn-gluconate to provide a ligand which will take up technetium-99m usually supplied in pertechnetate form. Suitable agents include glucohephonate, MDP, pyrophosphate and HIDA derivatives.

The kit is used by adding the sterile buffer from the second vial to the antibody vial. Pertechnetate is added to the imaging agent vial and a suitable volume of this material is then added to the antibody vial. The mixture is typically incubated for five to ten minutes allowing for quatitative transfer of technetium-99m from the imaging kit to the antibody. The resultant technetium-99m labelled antibodies are ready for injection into patients without further purification.

Use of the present invention permits a simple, readily controlled chemical reaction to be used for producing the

kit and the kit is relatively simple to use in practice. A high specific radioactivity technetium-labelled Fab' on a weight-for-weight basis is obtainable.

In another aspect, the invention extends to a kit for use in scintigraphic imaging of thrombi in mammals comprising the thiolated material produced in the method described in any one of the forms above and a supply of exchange complex in a form suitable for labelling with a radionuclide, the kit being in a form such that reaction of the thiolated material with the exchange complex (when labelled) produces a solution for injection into a mammal with or without further purification.

Investigations showed that reduced-thiolated Fab', or thiolated Fab' fragments retained their labelling ability and immunoreactivity after 3 months when immediately freeze dried after HPLC or gel column separation.

In yet another aspect, the invention consists in a method of scintigraphic imaging comprising using a kit as described above.

In the following discussion reference is made to the use of materials which are commercially available as indicated below. The antifibrin monoclonal antibody DD-3B6/22 and its $F(ab')_2$ fragment and fibrin D dimer were supplied by Agen Biomedical Pty. Ltd. (Brisbane, Australia). Dithiothreitol and immnoglobulins free bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St Louis). RM 6, a renal imaging kit consisting of calcium gluconate, stannous chloride and Tc99m pertechnetate were obtained from Australian Radioisotopes (Sydney, Australia). Biogel P-6DG was from Biorad. Sepharose 6 MB was purchased from Pharmacia (Uppsala, Sweden).

EXAMPLE 1

In this example there was prepared technetium-99m labelled thiolated intact monoclonal antibody. The method of production was as follows:

1. 0.1M ammonium bicarbonate containing 2mM EDTA was applied to 0.7 mg of intact antifibrin monoclonal antibody DD-3B6/22 and the mixture cooled on ice.

2. 33 µl of 0.25M 2-pyridinealdoxime methiodide in bicarbonate buffer and 33 µl of 0.25M N-acetyl homocysteine thiolactone also in bicarbonate buffer were added to the solution of step 1. Using 20% TRIS, the ph was adjusted to 9.0, the container was flushed with N_2 and the mixture was stirred gently for 2.0 hours, while the ph was checked and maintained at 9.0 every 30 minutes.

3. Purification of the mixture was effected at the end of the incubation period by centrifugal desalting on Biogel P-6DG equilibrated in 0.1M sodium acetate buffer at ph 5.6.

4. Renal imaging agent RM6 was constituted with 1.0ml of pertechnetate eluted from a technetium generator having radioactivity in the range 30 to 300 mCi/ml. A 0.1ml aliquot of this technetium-99m mixture was added to the thiolated antibody.

5. The mixture having a final protein concentration of 0.95 µg/ml was incubated at about 37°C. By known monitoring techniques, it was found that quantitative labelling (> 99%) of antibody can be achieved in under 15 minutes.

EXAMPLE 2

In this example a thiolated monoclonal antibody fragment Fab' was produced and radiolabelled as follows:

1. Antifibrin monoclonal antibody DD-3B6/22 was subjected to pepsin digestion to produce the $F(ab')_2$ fragment.

2. Dithiothreitol reduction was effected to produce the Fab' fragment, a reducing agent: antibody mole ratio of 12:1 being used to permit high radionuclide incorporation in the subsequent step. 1.0 mg of the fragment $F(ab')_2$ in phosphate buffer saline was incubated with 12.0 µl of a 10 mM solution of dithiothreitol in a final volume of 300 µl at

37 d g C for 30 mins. Exc ss reducing agent was removed by centrifugal desalting using Biogel P-6DG equilibrated with PBS. The reduced antibody was obtained in an undiluted form and used immediately.

Without being bound to any particular theory, the inventors suggest that this may be due to reduction of disulphide bridges around the antibody hinge region.

3. Thiolation of Fab' fragments with DL-acetyl homocysteinethiolactone was achieved using 2-pyridinealdoxime methiodide as catalyst.

4. Technetium-99m ligand complex was prepared by adding 2.5 µl of a mixture containing 20 µg of calcium gluconate and 0.5 µg of stannous chloride to 250 µl of pertechnetate.

5. The reduced and thiolated antibody fragment was incubated with 120 µl of this Tc99m/gluconate mixture for 10 mins at room temperature. Excess technetium-99m was removed by centrifugal desalting.

The extent of labelling was determined by gamma counting in a dosimeter (Nuclear Associates) and protein was determined by the method of Bradford. Specific radioactivity of up to 50 mCi/mg of antibody protein was obtained and this level was considered satisfactory.

6. Purification of the labelled antibody fragment by high pressure liquid chromatography (HPLC) was then effected.

Gel permeation high pressure liquid chromatography (HPLC) was performed on a biosil TSK-250 column (7.5 x 300mm) equilibrated in 0.2M Tris/HCI buffer pH7.2 at flow rate of 1.0 ml/min. Protein was monitored continuously by its U.V. adsorption at 280 nm while radioactivity was similarly monitored with a flow through gamma detector peaked to the 140 keV gamma emission of Tc99m. This testing demonstrated that adequate purification of the solution was achieved with removal of unlabelled antibody material and unreacted Tc-complex. The resultant liquid when adjusted to be isotonic was suitable for injection into a mammal.

Immunoreactivity of the labelled antibody was determined by a solid phase assay. It was found that a most satisfactory level of immunoreactivity of greater than 75% was achieved.

The stability of radiolabel on the antibody fragment was investigated. Freshly prepared, 24 and 48 hrs aged technetium-99m labelled Fab' antibody, as well as labelled antibody incubated in 10 mM DTPA at pH 7.0 for 30 mins at 37 deg centigrade were similarly analysed by HPLC. Results indicated that the label was stable on the antibody and there was no evidence of transchelation of technetium-99m to DTPA.

EXAMPLE 3

This example describes the preparation of thiolated Fab' lyophilised kits. The method was as follows:

1. 12.0mg of antifribin monoclonal antibody DD-3B6/22 fragment $F(ab')_2$ was mixed with 0.1M deaerated ammonium bicarbonate/2mM EDTA and the mixture cooled on ice.
2. The mixture was thiolated by the addition of 0.4ml 2-pyridinealdoxine methiodide and 0.4ml N-acetylhomocysteine thiolactone and the ph adjusted to 9.0.
3. The mixture was incubated on ice for two hours while maintaining the ph at 9.0.
4. The antibody mixture was purified by centrifugal desalting on Biogel P-6DG equilibrated in deaerated water to produce purified Fab' fragment.
5. The fragment was divided in 0.7mg lots and lyophilised and upon completion of freezed drying in vials, the vials were sealed in vacuum and stored at -20°C.
6. At a later date the freezed dried thiolated Fab' fragment was labelled with the first step comprising adding 0.3ml of a 0.1M solution of sodium acetate buffer at ph 5.6 to th fragment.

7. 0.3ml of technetium-99m gluconate was added to the fragment and the result mixture incubated at up to 37°C.

TESTS

The labelling efficiency was monitored and the results indicated that quantitative incorporation of technetium-99m into Fab' was complete in under 15 minutes and the free pertechnetate in the sample was under 0.5%. A comparison example was monitored in which the same antibody fragment was treated just with DTT reducing agent. Significantly at the labelling stage it took much longer to incorporate radioactivity and the maximum amount of incorporation was 97.5%.

Importantly, with the thiolated labelled Fab' fragment there was no evidence of recombination of Fab' to $F(ab')_2$. By contrast a corresponding experiment showed significant recombination of DTT produced Fab' back to $F(ab')_2$.

A further experiment was effected with a reconstituted lyophilised Fab' kit. Two UV absorbing peaks were found on elution representing $F(ab')_2$ and Fab' but elution to detect technetium-99m showed that only Fab' incorporated the technetium-99m. The predominant UV absorbing peak is the Fab'.

Immunoreactivity was also investigated and it was found that for labelled thiolated Fab' the immunoreactivity had 75% binding ability, a satisfactory result.

A further experiment was conducted by incubating a labelled thiolated Fab' fragment in serum at 37°C over a five hour period. By HPLC analysis, it was shown that radioactivity remained fairly well bound to the thiolated antibody.

Biodistribution and localisation experiment in rabbits was conducted. Comparisons were made between thiolated labelled Fab' and DTT generated labelled Fab'. Investigations were made four hours after injection and similar results were obtained for both the samples. Major

tissue uptake in the kidneys was found and this is consistent with rapid blood clearance, a desirable feature for scintigraphic diagnostic tests. Furthermore localisation of labelled Fab' to an experimental clot was excellent with a corresponding control region indicating little uptake.

EXAMPLE 4

In this example technetium-99m labelled thiolated proteinaeous material was produced for scintigraphic monitoring purposes. Preparation was as follows:

1. 25µg of serum amyloid proteins (SAP) in 0.1m ammonium bicarbonate/2mM EDTA buffer were treated with 4.0µl of N-acetylhomocysteine thiolactone (0.25M). The mixture was kept at 0-4°C overnight.
2. Purification of the thiolated SAP was effected by centrifugal desalting on Biogel P-6DG equilibrated in 20mM TRIS/HCL, pH7.2, 0.15 M NaCl.
3. 5.0µl of techetium-99m labelled, reconstituted renal imaging agent Rm6 was added to the thiolated SAP, to yield a final SAP protein concentration of 0.6 mg/ml. Incubation was effected at room temperature.

Investigation were made and results indicated a 90% labelling efficiency can be achieved.

Modifications and Variations

In the above example 2 as an alternative the antibody can be desalted in a column equilibrated with deoxygenated water instead of PBS.

In the above example 2 immediate use was indicated but most advantageously it has been found that freeze drying of the thiolated Fab' fragments and the gluconate complex in separate containers has b en successful. Reconstitution of these components followed by labelling of the gluconate with

technetium and then mixing of the two solutions resulted in an effective product.

Another alternative is to use gel column chromatography for purification of the antibody instead of HPLC.

Claims:

1. A method of producing a kit for scintigraphic detection of thrombi in mammals including humans, the steps comprising taking a material which is directed specifically against blood clots, the material being from the group consisting of a proteinaceous material, a monoclonal antibody, a single domain antibody, or an epitope binding fragment of a monoclonal antibody or a single domain antibody, conjugating the material with a thiolating agent, whereby there is provided a conjugated material adapted to be labelled with an acceptable radionuclide.the steps comprising taking a monoclonal antibody or a fragment thereof directed specifically against blood clots, and conjugating the antibody or fragment with a thiolating agent, and labelling the conjugated antibody or fragment with an acceptable radionuclide.

2. A method as claimed in claim 1 and wherein the selected material recognises the D-dimer (DD) epitope of human cross-linked fibrin.

3. A method as claimed in claim 2 and wherein the selected material is a monoclonal antibody identified herein as 3B6.

4. A method as claimed in any one of the preceding claims and wherein the method includes obtaining the $F(ab')_2$ fragment of a monoclonal antibody, and producing the thiolated Fab' fragment.

5. A method as claimed in claim 4 and wherein the Fab' fragment is derived from reduction of the $F(ab')_2$ fragment of the antibody by the use of dithiothreitol and this step is followed by thiolating.

6. A method as claimed in claim 4, and wherein the $F(ab')_2$ fragment is reacted directly with a thiolating agent to produce the Fab' fragment.

7. A method as claimed in any one of the preceding claims and wherein thiolation is effected using DL N-Acetylhomocystein-thiolactone.

8. A method as claimed in any one of the preceding claims wherein labelling of the thiolated antibody material is effected by exchange labelling with a labelled gluconate, or other complexes of intermediate association.

9. A method as claimed in any one of the preceding claims wherein the labelling is effected using the radionuclide technetium-99m.

10. A method as claimed in any one of the preceding claims and further comprising purifying the thiolated conjugate using gel column chromatography or high pressure liquid chromatography.

11. A method as claimed in claim 1 and wherein the method is effected by thiolating serum amyloid proteins.

12. A kit for producing an injectable material for use in scintigraphic imaging of thrombi in mammals, the kit comprising a thiolated antibody or fragment thereof as produced in the method claimed in any one of the preceding claims and a supply of exchange complex in a form suitable for labelling with a radionuclide, the kit being in a form such that reaction of the thiolated antibody or fragment with the exchange complex (when labelled) produces a solution suitable for injection into a mammal, with or without further purification.

13. A method of scintigraphic imaging in a mammal comprising taking the material as produced in any one of claims 1-11 or taking a labelled solution produced from the kit of claim 12, injecting the material into the mammal and scintigraphic imaging.

14. A method of producing material for use in scintigraphic imaging in mammals and substantially as herein described in any one of the Examples.

15. A kit for producing an injectable solution for scintigraphic imaging comprising a freeze dried thiolated material produced as described in any one of the Examples, and a supply of exchange complex suitalbe for labelling with a radiopharmaceutical and suitable for addition to the thiolated material when reconstituted.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00372

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] A61K 49/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	A61K 49/02, 43/00

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

CHEM ABS using keywords: ANTIBOD: + SCINTIGRAPH: + RADIONUCLIDE (S)

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X	EP,A, 318948 (NEORX CORPORATION) 7 June 1989 (07.06.89) See claims; page 7 lines 46-55; page 9 line 17 - page 10 line 19	(1-15)
X	WO 89/07456 (MALLINCKRODT, INC.) 24 August 1989 (24.08.89)	(1-15)
X	AU,A, 20685/88 (IMMUNOMEDICS, INC.) 16 February 1989 (16.02.89) See claims; page 20 lines 20-25; Example 6	(1-15)
P,X	WO 89/09405 (IMMUNOMEDICS, INC.) 5 October 1989 (05.10.89) See claims; page 5 lines 8-12	(1-15)
P,Y	WO 90/05544 (CENTOCOR, INC.) 31 May 1990 (31.05.90)	(1-15)

(continued)

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 October 1990 (25.10.90)	30 October 1990
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	JOHN G. HANSON

Form PCT/ISA/210 (second sheet) (January 1985)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	WO 88/07382 (CENTOCOR CARDIOVASCULAR IMAGING PARTNERS, L.P.) 6 October 1988 (06.10.88)	(1-15)
Y	US,A, 4434151 (BYRNE, E.F. et al) 28 February 1984 (28.02.84) See column 1 lines 1-50	(1-15)

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL APPLICATION NO. PCT/AU 90/00372

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
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END OF ANNEX